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A Ratiometric Fluorescent Probe for Cysteine and Homocysteine Displaying a Large Emission Shift

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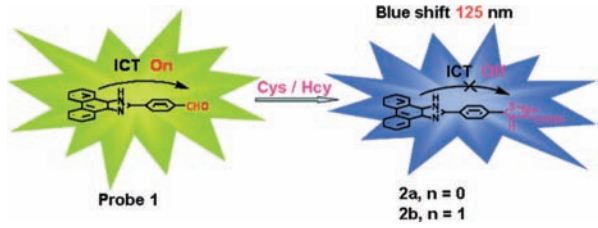
ABSTRACT



4-(1H-phenanthro[9,10-d]imidazol-2-yl)benzaldehyde 1 was rationally designed as a novel ratiometric fluorescent probe for cysteine and homocysteine. Upon addition of cysteine or homocysteine, notably, the probe displayed a very large (125 nm) hypsochromic shift in emission due to switching off intramolecular charge transfer. This large emission wavelength shift may allow probe 1 to be employed for quantitatively detecting Cys/Hcy.

Cysteine (Cys) and homocysteine (Hcy) play crucial roles in many physiological processes. For instance, an abnormal level of cysteine is implicated in diseases such as liver damage, skin lesions, slowed growth,[1] and an elevated level of homocysteine is a risk factor for Alzheimer's and cardiovascular diseases.[2] Thus, the detection of Cys/Hcy has been the subject of much attention. A number of fluorescent probes for Cys/Hcy have been developed recently.[3] However, most of these fluorescent probes response to Cys/Hcy with changes only in fluorescent intensity. A major limitation of intensity-based probes is that variations in sample environment and probe distribution may be problematic for utilization in quantitative measurements.[4] By contrast, ratiometric fluorescent probes allow the measurement of emission intensities at two different wavelengths, which should provide a built-in correction for environmental effects and can also increase the dynamic range of fluorescence measurement.[5]

The aim of our work was to develop a novel fluorescent probe which displayed a ratiometric response to Cys/Hcy. Ideally, the newly developed ratiometric fluorescent probe should exhibit a large emission wavelength shift (>80 nm)

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for practical use in ratiometric measurement.[6] Clearly, it is a great challenge to acquire such a probe for Cys/Hcy.

When a dye possesses an electron rich group conjugated to an electron deficient group, intramolecular charge transfer (ICT) from the donor to the acceptor will proceed upon excitation. ICT is an effective signaling mechanism employed in design of ratiometric fluorescent probes. For a probe functioning through ICT to undergo a spectral shift, the ICT efficiency of the probe should be modulated upon interaction with an analyte. One typical way to achieve this is to directly engage the charge of an analyte with the electron donating or withdrawing group of the probe in the binding process. However, due to the structures of Cys/Hcy, it is challenging to judiciously design such a ratiometric fluorescent chemosensor in the context of Cys/Hcy coordination. To circumvent this difficulty, we reasoned that, if reaction of Cys/Hcy to a probe can result in deconjugation of the electron donor-acceptor system, the ICT will be switched off. Thus a blue shift in absorption and emission spectra should occur.

Based on this strategy, in this work, we developed 4-(1H-phenanthro[9,10-d]imidazol-2-yl)benzaldehyde, compound **1** (Figure 1), as a new ratiometric fluorescent probe for Cys/Hcy. Upon introduction of Cys/Hcy, the probe displayed a drastic (125 nm) hypsochromic shift in emission. Probe **1** is composed of a phenanthroimidazole moiety and an aldehyde group. The electron rich phenanthroimidazole moiety was selected to act as both a fluorescent dye and electron donor[7] in the ICT process, which is the key to the success of this strategy. The aldehyde group was chosen for two reasons. First, it was expected to function as an electron acceptor in the ICT process, so it was conjugated to the phenanthroimidazole donor through a phenyl spacer for the effective ICT. Second, the aldehyde group has been known to react specifically to Cys and Hcy to afford thiazolidine and thiazine, respectively.[3a−c] Notably, in adducts **2a** or **2b**, the thiazolidine or thiazine group is deconjugated from the phenanthroimidazole moiety, so the ICT should be turned off. In the emission spectra, the ICT emission band should disappear, and a blue-shifted locally excited (LE) band should instead appear. It is worthy to note that, although the formation of thiazolidines or thiazinanes has been used in the Cys/Hcy probe development, it has not been employed in the context of modulation of ICT to design ratiometric fluorescent probes for Cys/Hcy.

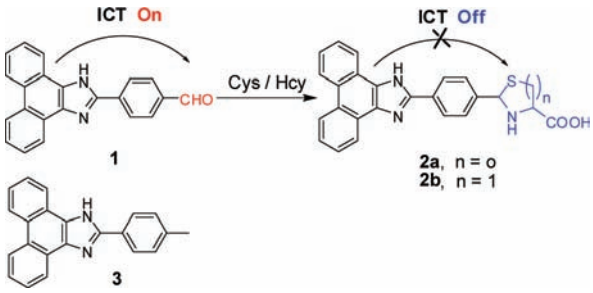

Figure 1. Design concept of ratiometric fluorescent probe **1** and the structure of the reference compound **3**.

Probe **1** was readily synthesized in one step by coupling 9,10-phenanthroquinone with terephthalaldehyde according to a reported procedure (Scheme S1),[8] and the reference compound **3** (2-p-tolyl-1H-phenanthro[9,10-d] imidazole, Figure 1) was also prepared by the same procedure (Scheme S1). In this work, the photophysical properties of probe **1** in the absence or presence of Cys/Hcy were studied in 10 mM HEPES buffer, pH 7.4/DMF (v/v, 1: 3) at ambient temperature. In comparison to the absorption spectrum of the reference compound **3**, that of probe **1** displayed a red-shifted and broad peak around 376 nm (Figure S1). This 46 nm red shift is apparently attributed to the ICT between the phenanthroimidazole moiety and the aldehyde group, as designed. The bathochromic shift due to ICT was also observed in the emission spectrum. Probe **1** exhibited a strong ICT fluorescence emission peak at 519 nm, which is bathochromic shift about 125 nm when compared to that of the reference compound **3** (Figure S2).

The optical response of probe **1** to various amino acids, glucose, and related thio-containing compounds was investigated by the UV−visible and emission spectroscopy. Probe **1** (2×10^{-5} M) was treated with 100 equiv of a series of amino acids, glucose, reduced glutathione (GSH), or 2-mercaptoethanol (MER) separately. As displayed in Figure 2a,

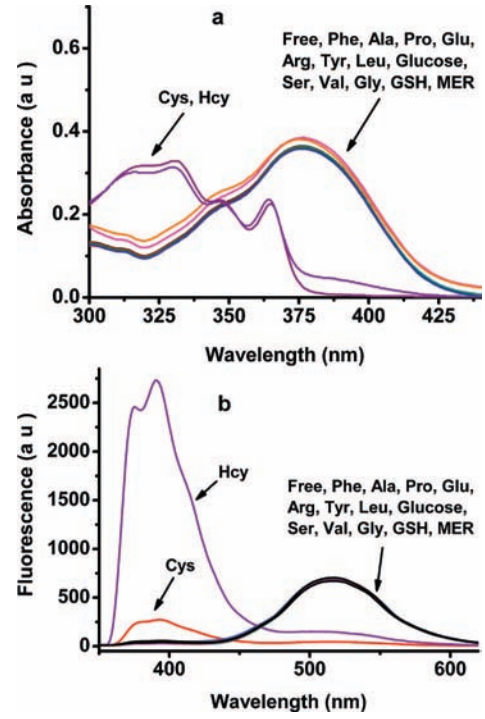

Figure 2. Absorption (a) and emission (b) spectra of probe **1** (2×10^{-5} M) with or without various amino acids, glucose, GSH, and MER (100 equiv).

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reaction of Cys/Hcy with the probe resulted in decrease of the absorption band around 376 nm and formation of a new hypsochromic absorption band with maximum at 330 nm. By contrast, no visible changes in absorption were observed upon addition of other amino acids, glucose, or thio-containing compounds, indicating that the aldehyde group specifically reacted with the β- and γ-aminoalkylthio moieties. In good agreement with the changes in the absorption spectra, Cys/Hcy elicited a large blue shift in the emission spectra (Figure 2b and Figure S3). However, no visible variations were observed upon addition of other amino acids, glucose, or thio-containing compounds, suggesting that the probe is highly selective to Cys/Hcy. Figure 3 shows the

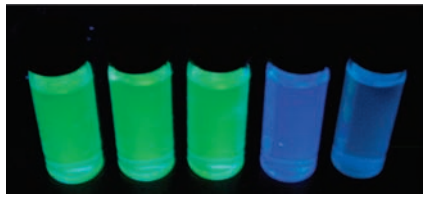

Figure 3. Visual fluorescence emissions of probe **1** to representative amino acids or thio-containing compounds on excitation at 365 nm using UV lamp at rt. (From left to right: probe **1**, probe **1** + Gly, probe **1** + GSH, probe **1** + Hcy, probe **1** + Cys.)

visual fluorescence emissions of probe **1** to amino acids or thio-containing compounds. The free probe exhibited a green emission color, and the addition of representative amino acids such as Gly or reduced glutathione did not change the color. By contrast, introduction of Cys/Hcy elicited a significant variation in emission color from green to blue. To further test the effective applications of compound **1** as a ratiometric fluorescent probe for Cys/Hcy, the fluorescence response of probe **1** to Cys/Hcy in the presence of typical competing species such as various amino acids, glucose, and related thio-containing compounds was studied. As shown in Figures S4−5, most of the competing species showed minimum interference in the detection of Cys/Hcy, indicating that probe

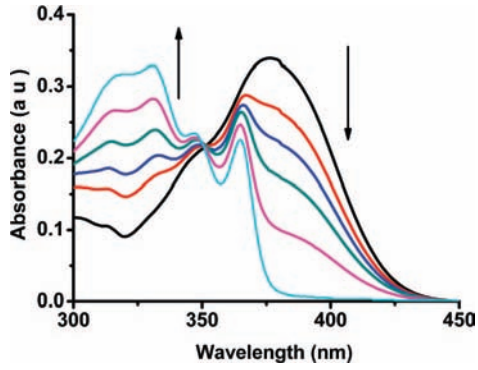

Figure 4. Absorption spectral changes of probe **1** (2×10^{-5} M) upon addition of Cys (0−100 equiv).

1 could be used for selective detection of Cys/Hcy even under competition from other related species.

Addition of an increasing concentration of Cys (0−100 equiv) to the solution of probe **1** (2×10^{-5} M) resulted in a gradual decrease of the absorption peak at 376 nm and a progressive increase of a new absorption peak around 330 nm (Figure 4). The marked blue shift signals that, indeed, the ICT is "off" in the adduct, in good agreement with the formation of thiazolidine. Similar hypsochromic shift was also found for Hcy (Figure S6). The high similarity between the absorption spectrum of the reference compound **3** and those of the probe titrated with Cys/Hcy corroborates that the ICT is off in the adducts. Consistent with the hypsochromic shift in the absorption spectra, addition of Cys/Hcy to the solution of probe **1** also caused blue shift in the emission spectra (Figure 5). With an increasing amount of

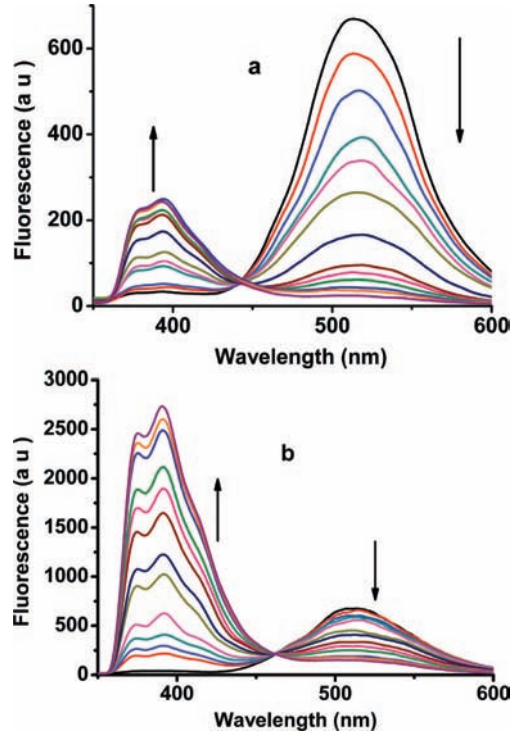

Figure 5. Fluorescence spectral changes of probe **1** (2×10^{-5} M) upon addition of increasing concentration (0−100 equiv) of Cys (a) and Hcy (b) ($\lambda_{ex} = 325$ nm).

Cys/Hcy, the intensity of the ICT emission band of probe **1** at 519 nm gradually decreased with the concomitant growth of a new LE band at 394 nm due to the adduct formation. The significant hypsochromic shift, up to 125 nm, enables the ICT and the LE emission peaks to resolve very well to afford a remarkable I_{394}/I_{519} change from 0.05 to 8.8 (an 176-fold enhancement) for Cys and from 0.05 to 46.0 (a 920-fold enhancement) for Hcy (Figure S3). The fluorescence quantum yields of the adducts **2a** and **2b** were determined to be 0.08 and 0.45, respectively (see the Supporting

Information).[9,10] The time course of the fluorescence spectra of probe **1** in the presence of Cys (Figure S7) or Hcy (Figure S8) indicates that there were significant spectral changes within minutes of addition of Cys/Hcy. The reaction essentially reached completion after 60 min, comparable to previous reports.[3b,c] An assay time of 60 min was chosen in the evaluation of the selectivity and sensitivity of probe **1** toward Cys/Hcy.

To confirm the formation of thiazolidine **2a** and thiazinane **2b**, probe **1** was treated with Cys/Hcy, and the reaction products were isolated. The partial ^{1}H NMR spectra of **1** and the isolated **2a** are shown in Figure 6. The resonance

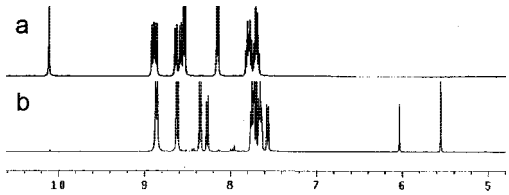

Figure 6. ^{1}H NMR spectra of (a) probe **1** and (b) the isolated thiazolidine derivative **2a** in DMSO-d_6.

signal corresponding to the aldehyde proton at 10.1 ppm disappeared; however, concomitantly, two new peaks at 5.55 and 6.02 ppm assigned to the methine proton of the thiazolidine diastereometer emerged, consistent with a previous report.[3a] The structure of thiazolidine **2a** was further characterized by the mass spectrometry analysis (Figure S9, Supporting Information). Furthermore, the isolated **2a** and the probe titrated with Cys have the identical absorption and emission spectra (Figure S10, Supporting Information). Similarly the ^{1}H, NMR, mass, and optical spectrometry analysis also support the formation of thiazinane **2b** upon treatment of the probe with Hcy.

As the probe showed a large emission wavelength shift upon treatment of Cys/Hcy, the ICT and LE emission bands are nearly completely resolved. This characteristic may allow precise quantitative detection for Cys/Hcy. To investigate this possibility, as an example, probe **1** (2×10^{-5} M) was treated with various concentrations of Cys, and the fluorescence was recorded at 30 min.[3c] The fluorescent intensity ratios at 394 and 519 nm were plotted as a function of the Cys concentration, and a typical calibration graph of the response to Cys under the optimum experimental conditions was obtained as shown in Figure 7. This plot shows a good linear relationship ranging from 0.6 to 80 $\times 10^{-5}$ M, essentially inclusive the physiological levels of Cys (the total concentra-

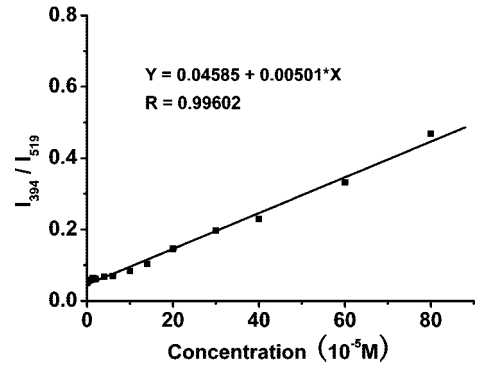

Figure 7. Plot of the fluorescent intensity ratios at 394 and 519 nm as a function of the Cys concentration.

tions of Cys in healthy plasma are typically in the range of 24 − 36 $\times 10^{-5}$ M).[3a] As shown in Table 1, ratiometric

Table 1. Determination of the Cys Concentration in the HEPES Buffer and the Newborn Calf Serum Solution

	Cys spiked (mol L^{-1})	Cys recovered (mol L^{-1})[a]	recovery (%)
HEPES buffer	0	not detected	
HEPES buffer	3.00×10^{-4}	$(2.96 \pm 0.03) \times 10^{-4}$	98.7
HEPES buffer	3.60×10^{-4}	$(3.45 \pm 0.03) \times 10^{-4}$	95.8
Serum	0	not detected	
Serum	3.00×10^{-4}	$(2.96 \pm 0.05) \times 10^{-4}$	98.7
Serum	3.60×10^{-4}	$(3.53 \pm 0.03) \times 10^{-4}$	98.1

[a] Relative standard deviations were calculated on the basis of three measurements.

fluorescent probe **1** was able to determine the concentrations of spiked Cys in the HEPES buffer and the new born calf serum solution with good recovery, indicating that probe **1** can potentially be employed for quantitatively detecting Cys.

In conclusion, compound **1** was developed as a novel ratiometric fluorescent Cys/Hcy probe. Importantly, upon introduction of Cys/Hcy, the probe displayed a remarkable blue shift (125 nm) in emission due to switching off ICT. This large emission wavelength shift in the ICT and LE emission bands may allow probe **1** to be employed for quantitatively detecting Cys/Hcy.

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Supporting Information Available: Synthesis of the probe and some spectra of the probe. This material is available free of charge via the Internet at http://pubs.acs.org.

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